Anxiolytic Effects of Chronic Treatment with Hypericum Perforatum (St John's Wort) Tincture in Male and Female Rats

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Abstract

Given high prevalence of anxiety disorders, it is important to identify effective alternative treatments due to the current limitations of pharmacological and psychological interventions. Worldwide, *Hypericum perforatum* (HP) products have become some of the best-selling herbal remedies. However, empirical evidence supporting their anxiolytic effects and investigations of dose levels are highly scarce. To begin empirical enquiry in this field, the present study sought to explore treatment effects of HP following chronic oral administration of an HP tincture in male and female rats. Several anxiety-related responses of 74 adult rats (35 males and 39 females) were studied in four behavioural testing apparatuses, including the open field test, the light/dark box, the y maze, and the elevated zero maze. Withdrawal responses were also investigated upon acute (72 hours) and chronic (14 days) HP treatment-free periods utilising the open field paradigm. Differences in anxiety responses were examined across sex and HP doses (0/mg/kg, 250mg/kg, 500mg/kg, and 750mg/kg). Results demonstrated evidence of anxiolytic effects of HP tincture and found that the 250mg/kg dose produced the greatest anxiolytic effect and the fewest withdrawal responses. Possible withdrawal responses were evident during the acute HP tincture treatment withdrawal period, pointing to possible anxiogenic effects upon acute discontinuation. Findings also showed sex differences, however, insufficient evidence was obtained from the present study with respect to a possible interaction between dose and sex. Despite the discussed limitations, the present study marked an important first step in initiating research into anxiolytic effects of commercially-popular herbal remedies and provides a foundation for further research into dose explorations, as well as dose and sex relationships.

Anxiolytic Effects of Chronic Treatment with Hypericum Perforatum (St John's Wort) Tincture in Male and Female Rats

Anxiety disorders are being diagnosed at alarming rates. An estimated 7.3% global prevalence rate of anxiety disorders has been established, which illustrates that one out of 14 people experience distress from anxiety disorders (Baxter, Scott, Vos, & Whiteford, 2013). Anxiety and related disorders are also the most prevalent among all psychiatric conditions (Kessler et al., 2005). In New Zealand, a 2012/2013 health survey reported more than 200,000 New Zealand adults suffer from anxiety disorders, which is 6.1% of the adult population (Mental Health Foundation, 2014). Sex differences have also been noted in diagnosis rates, with women being 1.6 times more likely to have anxiety disorders in comparison to men (Fairbrother, Janssen, Antony, Tucker, & Young, 2016). In New Zealand, 7.7% of women have been diagnosed with an anxiety disorder compared to 4.4% of men (Mental Health Foundation, 2014). These statistics establish the urgent need for adequate forms of treatment. However current pharmacotherapy is still limited in its effectiveness (Kirsch et al., 2008; Turner, Matthews, Linardatos, Tell, & Rosenthal, 2008). The usual medications, such as selective serotonin reuptake inhibitors (SSRIs) and benzodiazepines are reported to have disabling side effects including dependence, memory impairment, and sedation (Pittler & Ernst, 2003), whereas psychological interventions are at a much higher cost. Due to the limitations of current treatments, there is a rising need to investigate the use of alternative remedies to combat anxiety disorders especially in view of increasing public awareness of alternative therapies such as herbal remedies (MacLennan, Wilson, & Taylor, 2002).

1.1 Hypericum Perforatum (HP)

Hypericum perforatum (HP), also known as St John's Wort, has been a pharmaceutical aid for more than two thousand years (Istikoglou, Mavreas, & Geroulanos, 2010). With its alleviating properties, HP has been used extensively in Europe as an antidepressant, antiseptic and anti-inflammatory agent, an expectorant and a tonic for the immune system. It is mostly studied and used for treating depression, anxiety, and sleep disorders in German-speaking countries (Linde, Berner, & Kriston, 2008a). In Germany, more than 100 million daily doses were prescribed in 2006, making HP one of the most widely used antidepressants (Wurglics & Schubert-Zsilavecz, 2006).

The complex and diverse pharmacological components of HP are still being investigated. The yellow flowers of HP contain at least ten classes of biologically active detectable compounds including naphthodianthrones (hypericin and pseudohyoericin), anthraquinones, carotenoids, cumarine, phloroglucinols (3% hyperforin), flavonoids (hyperoside, quercetin and rutin), proanthocyanidins, tannins, amino acids, xanthones, volatile oils, and other water-soluble components (Greeson, Sanford, & Monti, 2001; Mullaicharam & Halligudi, 2019; Nahrstedt & Butterweck, 2010). Its antidepressant effects are explained by a combination of pharmacological mechanisms similar to standard antidepressants. However the pharmacology of HP is not yet fully understood. Multiple mechanisms may be involved in its antidepressant action, such as effects on serotonegic, noradrenergic, and dopaminergic systems, as well as GABA and glutamate amino acid neurotransmitters (Beckman, Sommi, & Switzer, 2000; Butterweck, 2003; Sarris, Panossian, Schweitzer, Stough, & Scholey, 2011). HP's currently identified components (hyperforin, hypericin, and groups of flavonoids) have been reported to account for the substance's neurochemical modulation (Butterweck & Schmidt, 2007).

Studies have shown that HP extracts are well-tolerated and have adverse reactions comparable to placebo (Ernst, Rand, Barnes, & Stevinson, 1998). The most frequently reported of these adverse effects comprise gastrointestinal irritations, allergic reactions, fatigue, restlessness, dizziness/confusion, tiredness/sedation and dry mouth (Ernst et al., 1998; Woelk, Burkard, & Grunwald, 1993). However they are generally mild, transient, and similar to placebo, if they do occur (Melchart, 1996), and the frequency of the occurrence is reported to be 2% (Schmidt & Sommer, 1997, as cited in Greeson et al., 2001).

1.11 HP Tincture

HP supplements have been repeatedly listed as one of the top best-selling herbal remedies worldwide. Different forms of HP products are sold on the current market including liquid (ethanol or oil) tinctures, herbal teas, and dry flowers. However, no specific form of HP extracts has been investigated with respect to its antidepressant effects. The current body of research, although relatively vast, is limited in only using the pure form of HP extracts such as LI160 without considering the investigation of HP products that are currently available on the market. Investigating a particular form of the product is necessary to ascertain effectiveness of HP extracts and to protect consumer safety. In terms of having good stability of the constituents and low microbiological contamination, tinctures are regarded as one of the best liquid forms of plant extracts. Two different alcoholic degrees (40% and 60% v/v) of HP tinctures have been studied, with results yielding different levels of flavonol and hypericin contents, which were attributed to different decomposition percentages (Bilia, Bergonzi, Mazzi, & Vincieri, 2002). This finding indicates that the effectiveness of HP tinctures may vary depending on the form and preparation of the HP extracts.

1.2 Antidepressant Effects of HP

HP has been traditionally used in the treatment of depression. It is one of the few herbal remedies with a body of research supporting its clinical use. Although the actual mechanism of HP's antidepressant activity has not yet been established, *hypericin*, one of the main HP constituents, is strongly associated with its antidepressant effects (Mullaicharam & Halligudi, 2019).

Other findings include specific cognitive and neural processing abnormalities, such as negative biases in emotional processing, are thought to be a major factor in the maintenance of depressive symptoms. Research with depressed populations has shown attentional biases towards negative cues and enhanced memory for negative contexts, such as words and facial expressions, rather than positive information (Leppänen, 2006). The acute administration of an antidepressant was reported to be effective in the modification of emotional processing (Harmer D Phil et al., 2009). Similar to this finding, a study by Warren, Cowen, and Harmer (2019) demonstrated reductions in negative attentional biases and improved memory for positive materials with HP treatment, thereby pointing to its antidepressant potential.

An early review Lieberman (1998) suggested that HP was safe and effective compared to tricyclic antidepressants (TCAs) for treating various forms of mild to moderate depression, without significant side effects and negative drug interactions. A meta-analysis conducted by Kim et al (1999) compared HP to TCAs and placebo. In the short-term treatment of mild to moderately severe depression, HP had similar effects to low dose TCAs, and was 1.5 times more likely to result in an antidepressant response than placebo. In addition, TCAs were reported to be more likely to cause side effects than HP. Effectiveness of HP was further supported by similar findings from Linde et al (2008) as well as Rahimi et al (2009). In their meta-analysis, Linde et al (2008) concluded that there is considerable

research evidence from German-speaking countries favouring the effectiveness of HP as an antidepressant. However, this has not been established outside of these countries.

1.3 Anxiolytic Effects of HP

Mood and anxiety disorders are the most prevalent psychological conditions, which often co-occur. It is estimated that half of individuals with depression also suffer from some forms of anxiety disorders (American Psychiatric, American Psychiatric, & Force, 2013). Currently, the only herbal remedy to be convincingly reported as having anxiolytic properties is *Piper methysticum*, also known as kava (Pittler & Ernst, 2003). However due to its association with hepatotoxicity, kava is currently not in clinical use and is restricted in several countries including the UK (Ernst, 2002).

It is suggested that HP may have anxiolytic effects by effectively altering the functioning of neurotransmitters such as serotonin, dopamine, GABA, and norepinephrine. In addition to its use as an antidepressant, HP has also been used for anxiety disorders such as generalised anxiety disorder. However there is yet no empirical support for its anxiolytic effects (Sarris, 2013). Previous studies have focused on its application in treating depression, but few studies have investigated the use of HP in the treatment of anxiety disorders. Vandenbogaerde et al. (2000) reported some limited evidence of anxiolytic effects of HP extract by testing rats' locomotor behaviour in the open field and in the light/dark box. The authors concluded that the potential anxiolytic activity resulted from the total extract rather than from a single component, such as protohypericin and a fraction containing hypericin and pseudohypericin. Additionally, Flausino, Zangrossi, Salgado, and Viana (2002) found some evidence of the HP's therapeutic potential in anxiety by observing rats in three different anxiety tests (elevated T-maze, arena, light/dark box, and cat odour test) after acute and chronic treatments (14 consecutive days) of the HP extract, LI160. Empirical research by

Kumar, Garg, and Prakash (2010) also suggested that HP could be used in the treatment and management of stress-related conditions. They reported improved locomotor activity, reduced tail flick latency and anti-anxiety-like effects in mice. In a recent study from Rojas-Carvajal, Fornaguera, Badilla, Brenes, and Calvo (2017), the anxiolytic effects of HP and diazepam were compared by inducing stress in anxious and depressive-like phenotype rats. The authors have found HP's anxiolytic effect to be equivalent to diazepam, and concluded that HP may be an alternative anxiety treatment with fewer side effects, and with a wider safety margin.

Since HP has been regarded as a herbal medicine rather than a health supplement, it is important to address its quality, efficacy, and safety. Consumers tend to use HP to treat stress, anxiety, and depression, believing it is a natural and safer option with fewer side effects compared to the standard antidepressants (Lewis, Willis, Kokanovic, & Pirotta, 2015). Although the majority of herbal products are reasonably safe to use (Posadzki, Watson, & Ernst, 2013), it is suggested that preparations of commercial HP products should be regulated and standardised (Bilia, Gallori, & Vincieri, 2002; Sarris, 2012). Problems can arise from variations in potency and number of ingredients in different brands and batches, and the general lack of regulation surrounding the use of HP products and appropriate doses (Ng, Venkatanarayanan, & Ho, 2017). Several meta-analyses have also emphasised the need for studies outside of German-speaking countries (Linde et al., 2008a; Rahimi, Nikfar, & Abdollahi, 2009). Furthermore, for the general use of HP there is still a lack of adequate information about its efficacy and application at different dose levels (Butterweck, 2003). Therefore, HP requires further research into its possible value as an anxiolytic agent.

1.4 Dosage and Withdrawal

1.41 Dosage

Compared to the major focus on HP's antidepressant effects, empirical investigations aimed at identifying the optimal dose levels for anxiety are lacking. The recommend dosage by the German Commission E was 2 - 4 g of the powdered HP herb which contains 0.5 – 3 mg of hypericin (Linde et al., 1996). However to date, there was no clear identification of the precise HP dose efficacy and its constituents. Few studies investigated the dose level efficiency or provided clear guidelines of the precise dose level(s). As previously mentioned, Flausino et al. (2002) tested rats' anxiety responses upon a 14-day consumption of HP extract (LI 160), and compared the efficiencies of the three dose levels of 62.5, 125, 250, and 500 mg/kg respectively. Their study concluded that the chronically treated rats with the dose level of 250mg/kg presented anxiolytic effects comparable to the reference drug imipramine. Moreover, a study from Beijamini and Andreatini (2003) tested doses of 150 and 300mg/kg of HP through repeated administrations over 21 days in male mice. They found anxiolytic-like and anti-panic-like effects for the 300mg/kg dosage group but not for the 150mg/kg dosage group. Another study from Grundmann, Lv, Kelber, and Butterweck (2010) investigated doses of 125, 250, 500, and 750 mg/kg body weight in male rats for 21 days. The authors reported that doses of 250, 500, and 750mg/kg significantly reduced the rats' stress levels. Furthermore, Negres et al. (2016) examined both acute and subacute toxicity of hyperforin (a major component of HP) in mice with 2000 and 5000mg/kg once per day, and 50, 75 and 100 mg/kg for 28 days, respectively. No lethal effect occurred in the acute toxicity tests. The authors stated that in a single dose, hyperforin was classified as Class V toxic: LD50>5000mg/kg. The toxic level of hyperforin for mice has been established as a median lethal dose (LD50) of > 5000 mg/kg, and thus classified as Class V toxic (Negres et al., 2016). Oral administration of a standardised 50% ethanolic extract of 100 and 200 mg/kg of

Indian *H. perforatum* once daily for three days in rat models of learning and memory resulted in significant attenuation of scopolamine and sodium-nitrite-induced impaired retention of active avoidance (Kumar, Singh, Muruganandam, & Bhattacharya, 2000). Overall, in order to gain further understanding of the effectiveness of HP at different dosages, based on previous research, this study will define dose strengths as low (250mg/kg/day), medium (500mg/kg/day), and high (750mg/kg/day).

1.42 Adverse and withdrawal effects

HP has been reported to have a favourable side effect and withdrawal profile. Studies have shown that HP extracts are well-tolerated and have adverse reactions comparable to placebo (Ernst et al., 1998). The most frequently reported of these adverse effects comprise gastrointestinal irritations, allergic reactions, fatigue, restlessness, dizziness/confusion, tiredness/sedation and dry mouth (Ernst et al., 1998; Woelk et al., 1993). However they are generally mild, transient, and similar to placebo, if they do occur (Melchart, 1996), and the frequency of the occurrence is reported to be 2% (Schmidt & Sommer, 1997, as cited in Greeson et al., 2001). The adverse effects reported are mild and mostly unspecific apart from skin reactions from HP induced photosensitivity from bright light (Klepser & Klepser, 1999; Knüppel & Linde, 2004).

It was noted that HP had fewer adverse incidents than synthesised antidepressants without evidence of dependency or abuse (Sarris & Kavanagh, 2009). To date, information regarding to the withdrawal responses of HP treatments is limited. A survey study from Beckman et al. (2000) found several standard antidepressants (paroxetine, fluoxetine, nortriptyline, desipramine, amitriptyline, and phenelzine) produced a similar withdrawal syndrome compared to the HP treatment that occurred during the first week of discontinuation. The withdrawal reactions included nausea, headache, increased anxiety,

irritability, dizziness, moodiness, tiredness, sleep difficulties, and influenza-like symptoms. However any withdrawal symptoms in the study were not related to either the dose or duration of use for either HP or the antidepressants.

There is no literature currently available for providing information on any withdrawal effects of HP tincture treatments. Therefore previous studies of the withdrawal effects of antidepressants and other substances provide a foundation for the current research regarding to the considerations of the HP treatment free time length and assessment methods. Some rats studies investigated the withdrawal effects of chronic antidepressant treatments such as fluoxetine assessed the rats' behaviours and neurobiological functions following 72 hours, 12, and 14 days substance-free periods (Lin, Koob, & Markou, 1999; Stolz, Marsden, & Middlemiss, 1983). Another study withdrawal effects of antidepressants on rats' circadian activity rhythms from Wollnik (1992) also adopted a 14-day of drug-free period. Furthermore, previous studies also assessed dose and withdrawn effects of amphetamine and progestone in rats at 7-, 14-, and 28-day periods and analysed the measures of exploratory behaviours including transitions, rearing, and centre square occupancy utilising the open field test (Hitzemann, Tseng, Hitzemann, Sampath-Khanna, & Loh, 1977; Löfgren, Johansson, Meyerson, Lundgren, & Bäckström, 2006).

From the available literature of withdrawal effect investigations, the present study will include an open-field test following 72 hours and two weeks of HP treatment-free periods respectively. However, it is important to note that currently there is no concern around potential side effects of HP treatments. As previously discussed, several meta-analyses have concluded that the rates of adverse events are comparable to placebo and lower than standard antidepressant treatment in human patients.

1.5 Sex Differences

Since none of the reviewed studies have involved both males and females, there is no information about any sex-related differences in the effects of HP. Sex-based dimorphisms naturally occur in brain anatomy, function and neurochemistry (Jazin & Cahill, 2010). Sex-related differences have also been found in clinical and preclinical investigations of the metabolism, distribution, and efficacy of antidepressants (Sramek & Cutler, 2011). Hormones can be responsible for triggering depressive episodes, and the fluctuations in female hormones may create a vulnerability for depression and anxiety for females (Parry, 1989; Seeman, 1997). Hughes (2019) recently surveyed publications of five journals within a 20-month period and emphasised the need to include both sexes for studies in behavioural pharmacology as female subjects were excluded from most of the studies. Thus, it is essential that possible sex differences are considered in studies of mood disorders.

There have been a number of attempts to explain why there are sex differences in depressive susceptibility, but a clear reason has not yet been determined (Sramek & Cutler, 2011). One plausible explanation is that female hormones are responsible for triggering depressive episodes, and the fluctuations in female hormones are creating a vulnerability for depression and anxiety for females (Parry, 1989; Seeman, 1997). Other suggestions involve sex differences in monoamine functioning and processing (Moreno, McGahuey, Freeman, & Delgado, 2006); sex differences in neurotransmitter systems such as the serotonergic system (Legato, 1996; Nishizawa et al., 1997) and the dopaminergic system (Laakso et al., 2002), and acknowledged differences in cognition between men and women (Hankin & Abramson, 2001).

A number of sex differences have been observed in the pharmacokinetic and pharmacodynamics actions of antidepressants (Keers & Aitchison, 2010). Therefore, both

males and females should be included in studies of the clinical efficacy of prescribed antidepressants. Several years ago it was announced by the U.S. National Institutes of Health that research experiments must include both sexes (Clayton & Collins, 2014). A number of studies of effects of antidepressants and other substances have shown sex differences in responsiveness to the drugs as well as their effects on brain functioning and metabolism, thereby supporting the need for including both sexes in psychopharmacological investigations (Biegon & Samuel, 1979; Carrier & Kabbaj, 2012; Gray & Hughes, 2015; Hughes & Hamilton, 2018; Hughes & Hancock, 2017).

1.51 Sex Differences in Human Studies

Studies have suggested that women may experience depression more severely and suffer greater functional impairment compared to males (Angst & Dobler-Mikola, 1984; Cahalane, Keller, & Hughes, 1994). Research has shown that depressed women tend to experience stronger appetites and thus greater weight gain, higher levels of anxiety, somatisation, anger, and more psychomotor retardation then men (Frank, Carpenter, & Kupfer, 1988; Kornstein et al., 2000; Perugi et al., 1990; Winokur, Coryell, Keller, Endicott, & Akiskal, 1993; Young, Scheftner, Fawcett, & Klerman, 1990).

With respect to the efficacy of particular antidepressants among men and women, numerous studies have found significant sex differences in therapeutic responsiveness. Women generally have a higher proportion of body fat and adipose tissue than men, which may result in a wider drug distribution for lipophilic antidepressants (Yonkers & Brawman-Mintzer, 2002; Yonkers, Kando, Cole, & Blumenthal, 1992). It is therefore reasonable to expect that women may require lower doses than men, or may have more adverse effects from antidepressants. Men and women also differ in their responses to the specific type of medication. An early study from Davidson and Pelton (1986) compared TCAs (imipramine

and amitriptyline) and MAOIs (phenelzine and isocarboxazid) among male and female patients with major depression, generalised anxiety, and panic disorder. For panic attacks, women who were treated with MAOIs responded more favourably than men, whereas men achieved better outcomes with TCA treatment. Overall, research findings illustrate the clear existence of sex-specific differences in the effectiveness of antidepressants.

1.52 Sex Differences in Animal Studies

Many animal studies show sex-specific effects of certain types of antidepressant in various brain regions. These differences have been attributed to sex differences in drug absorption, bioavailability, drug distribution, metabolism, and elimination. Human studies also suggest some similar mechanisms for sex differences during antidepressant treatment. There is a reasonable volume of research demonstrating sex dimorphism in response and adaptation to stress in rats (Dalla et al., 2008; Drossopoulou et al., 2004; Kamper et al., 2009). Specific focus has been given to exploring how antidepressants act on the stimulation of synaptic plasticity and neurogenesis enhancement (Kuipers et al., 2006; Nibuya, Nestler, & Duman, 1996), which may differ between males and females.

In a study by Gray and Hughes (2015) there was evidence of sex-specific effects on anxiety and memory in rats following over 21 days of chronic antidepressant treatment. This evidence consisted of dose-dependent and sex-specific effects on anxiety of the SSRI fluoxetine and the SNRI venlafaxine, whereas the NRI reboxetine was ineffective. For venlafaxine-treated males and females, low dosage in male rats showed anxiogenic effects through displaying higher rates of grooming behaviour while an anxiolytic effect was possible for females because of their decreased grooming. Although open-field behaviour did not show a clear pattern in sex differences, there were significant reductions in ambulation and rearing behaviours for fluoxetine-treated female rats only. In addition, for males only,

short-term spatial memory impairment may have resulted from all three drugs because of their impaired ability to recognise a novel change in a Y maze. This latter finding is consistent with previous male-specific findings, such as impaired inhibitory avoidance in mice and rats, as well as impaired spatial memory in rats due to fluoxetine and venlafaxine treatments (Monleón, Urquiza, Arenas, Vinader-Caerols, & Parra, 2002; Sass & Wörtwein, 2012; Ulak et al., 2006).

1.6 Animal Research and Behavioural Tests

Animal research is important for discovering new anxiolytic medications and for further understanding the neural basis of anxiety (File, 1984). The most commonly applied HP treatments in animal studies use the manufactured product of HP extract, LI160, which is currently not obtainable outside of Germany. However, none of the previous studies employed any commercially available HP products which are most commonly purchased and consumed by patients in the general public for mood disorders. Therefore, this present study made use of an HP tincture which is commercially manufactured and available from a local New Zealand supplier. The investigation was conducted with laboratory rats making use of a combination of four ethologically-based "unconditioned" behavioural tests (i.e., the open-field, zero-maze, Y-maze, and light/dark box).

These tests are designed to observe animals' fear and anxiety in response to mildly stressful stimuli which do not explicitly involve pain or discomfort. In addition, they minimise the risk of confounding effects in the experiment caused by motivational or perceptual states such as hunger and learned behaviour (M. Bourin, 2015). The unconditioned response tests, including open field, zero-maze, and light/dark box, require no training and usually have a high eco/ethological validity (Steimer, 2011). Multiple tests are employed to assess each individual rat's behavioural phenotype, since these tests measure anxiety under

different conditions (van Gaalen & Steckler, 2000). Data obtained from different tests can be combined to create "derived" variables which offer a more complete description of the individual behavioural profiles (Steimer & Driscoll, 2005).

The chosen behavioural tests involved recording open field responses, light/dark box activities, Y maze arm occupations, and elevated zero maze transitions. For example, in open field testing, higher levels of anxiety are indicated by behavioural observations such as increased latencies to emerge, higher levels of grooming, corner occupancy, and defecation. In contrast, measures such as higher frequencies of centre occupancy, walking, and rearing are associated with lower levels of anxiety (Archer, 1973; Brain & Marrow, 1999). These tests have the advantages of being simple and do not require prior animal training (Michel Bourin & Hascoët, 2003).

1.7 Aim of the Present Research

A major goal of the present study was to investigate treatment effects of HP on several anxiety-related responses following chronic oral administration of an HP tincture. This was done by comparing rats' anxiety responses on four behavioural tests among the HP tincture treatment groups and the control group. Both male and female rats of the same age were included in the study with approximately even numbers of each in order to explore possible sex differences in responses. Unlike previous studies conducted in rats that used oral gavage, the present investigation used a non-invasive method for chronic oral administration (21 days) by mixing HP tincture with rats' drinking water. The 21-day period was selected in accordance with a study by Grundmann et al. (2010) of chronic restraint stress in rats. This study also aimed at identifying the most effective doses(s) of the HP tincture in order to provide some guidance for any clinical use of this product, or for self-medication. This was

done by testing the HP tincture's anxiolytic effects with three target doses, namely, this study low (250mg/kg/day), medium (500mg/kg/day), and high (750mg/kg/day).

It was hoped that results from the study might highlight any the anxiolytic potential of HP commercial products for both sexes, determine dose-related effects, and evaluate the safety of the product by observing any occurrence of withdrawal symptoms. In sum, the present study aimed to examine the anxiolytic potential of commercial HP products, compare its effects across sexes and different dose levels, and evaluate withdrawal symptoms, thereby providing further empirical evidence of the utility and safety of HP products.

1.8 Predictions

Four hypotheses were generated for the present study. The first goal of the study was to investigate the anxiolytic effects of the HP tincture. Thus, Hypothesis 1 predicted that anxiety responses will be lowered among rats in the HP treatment groups compared to the control group.

The second aim of the study was to assess HP treatment effects with three doses so as to potentially detect an optimal dose level. Therefore, Hypothesis 2 predicted that there will be different patterns of behavioural responses among the dose groups, and that there will be evidence of which dose(s) produce the greatest reductions of anxiety.

The third goal was to assess the overall dose and sex interaction effects among all the groups. Thus, Hypothesis 3 predicted that there will be sex differences in how each dose affects anxiety.

The final aim was to determine whether any withdrawal effects occur following the 21 days of chronic administration. Thus, Hypothesis 4 predicted that, following treatment discontinuation, withdrawal responses will be greater for rats in the HP treatment groups compared to those in the control group.

Method

2.1 Subjects

Subjects were 40 male and 40 female PVG/c hooded rats bred in the Animal Facility of the University of Canterbury's Department of Psychology, Christchurch, New Zealand. The minimum number of 80 rats was determined from the result of a statistical power analysis for a 4 x 2 factorial ANOVA design. Forty-six of the rats (20 males and 16 females) were 12 days older than the remaining 44 rats (20 males and 24 females). Because of heavy demands for rats and cages required by other researchers, to achieve sufficient numbers for the present study this slight age difference was unavoidable and beyond the control of the writer. A total of six rats refused treatment consumption (five males and one female), and were therefore excluded from the study.

The procedures and methods used in this study, including the housing, feeding, treatment, doses, procedure, and testing apparatuses, were approved by the Animal Ethics Committee of the University of Canterbury.

2.2 Housing and Feeding

All rats were weaned at 30 days after birth (PN 30) and housed in same-sexed pairs in opaque plastic cages measuring 560 mm x 350 mm x 215 mm (length x width x height). At either PN77 or PN65, the pairs were separated by means of a 560 mm x 215 mm (length x height) wire mesh partition. The identification of each rat was enabled by spraying one rat of the pair on the same side of the cages with a non-toxic, coloured branding spray. This housing method prevents physical contact. However it allows visual, olfactory, and auditory communication, thereby minimising any detrimental effects of social isolation. Both rats shared a common ad libitum food dispenser (commercial rat pellets). A glass bottle holding approximately 250 ml of drinking solution was provided for each rat in order to ensure the

administration of specific treatment dosage. All rats were given continuous access to their appropriate drinking solutions on PN 97 or PN 85 for 21 days. The housing environment was maintained at an ambient temperature of 22 ± 2°C and humidity of 48 ± 10% on a 12-hour light/dark cycle (lights on at 08:00).

2.3 HP Treatment and Doses

The HP drinking solutions consisted of HP tincture, plain tap water, and sucrose. The water-soluble HP tincture (60% ethanolic 1:2 tincture) was purchased from Weleda, a New Zealand local manufacturer for natural and organic products. Dose levels were based on previous literature describing the effects of HP on anxiety-related behaviour (Beijamini & Andreatini, 2003; Grundmann et al., 2010). To achieve approximate daily doses of 250, 500, and 750mg/kg, the quantities of HP tincture to be added to drinking water were calculated from records of the rats' daily water consumption and body weights. Because male rats drink relatively less water in comparison to female rats (Hughes, Lowther, & van Nobelen, 2011), these quantities were determined separately for each sex. For male rats, the volumes of HP tincture that needed to be contained in each litre of drinking water to achieve the target daily doses for each HP treatment group were 8.3, 16.6 and 24.8 ml respectively. For female rats, these volumes were 6.3, as.5 and 18.8 ml.

In order to encourage consumption, 1% of sucrose was added to the drinking solutions for all groups to increase their palatability. Table 1 presents the number of male and female rats in each of the four treatment groups (control and low, medium, and high dose groups). Once a week each rat's bodyweight and consumption of its drinking solution were recorded to monitor its daily HP dose. All drinking bottles were then sterilized and refilled with clean solutions.

Table1.

Number of rats and dose groups

	Control (Plain water)	Low Dose HP (250mg/kg)	Medium Dose HP (500mg/kg)	High Dose HP (750mg/kg)	Total
Number of Male Rats	10	9	9	7	
Number of Female Rats	10	10	10	9	
Total	20	19	19	16	74

2.4 General Procedure

All behavioural testing took in place following 21 successive days of HP treatment (PN106 or PN118) when the rats had reached adulthood (Andersen, 2003). The rats were individually observed in four types of apparatus (an open field, Y maze, light/dark box and zero maze) with at least one day between each test. Upon the completion of all behavioural testing, the HP drinking solutions were replaced with plain water containing 1% sucrose. Following a 72-hour and 14-day HP treatment-free period respectively, possible withdrawal effects were investigated by performing open field tests. Random assignment and counterbalancing (Balanced Latin Square) were applied to ensure all the rats experienced the tests in different orders. In addition, the same behavioural tests were conducted during the same time period each day for the rats in all groups. For example, all of the open-field tests were conducted from 1pm to 9pm. After a rat's test, faecal boluses were removed from the apparatus which was then cleaned with a 4% solution of Express Sani (a commercial detergent sanitiser).

Each type of testing apparatus was illuminated by overhead fluorescent room lighting of 623lx, 345lx, 406lx, and 21lx for the open field, light/dark box, Y maze, and zero maze respectively.

2.5 Testing Apparatus and Behavioural Measures

Anxiety-related (and other) responses were recorded in each type of apparatus by means of a momentary time-sampling procedure that involved noting the rat's behaviour every 3 seconds. Each 3-second period was indicated by an auditory signal delivered through an ear-piece. Where appropriate, latencies of responding were recorded with a stopwatch, and entries into different parts of the apparatus were manually counted.

2.5.1 Open Field

The open field has become one of the most popular and convenient types of apparatus for measuring anxiety-like behaviours in animal research (Prut & Belzung, 2003). Prut and Belzung (2003) stated that the open field could trigger rodents' agoraphobia and anxiety due to separation from their conspecifics. It is therefore a pertinent measure for measuring anxiety in rats as they are social animals and prefer to occupy small rather than large areas.

The open field comprised a square 635 mm x 635 mm x 270 mm high wooden box without a lid which was painted black. A 200 mm x 150 mm x 195 mm (length x width x height) small wooden start box was attached to the open field with a hinged lid. The floor of the open field was divided into 16 equal-sized squares by means of a grid of intersecting white lines. The squares were numbered from 1 to 16. The corner squares were numbered 1, 4, 13, and 16, whereas numbers 6, 7, 10, and 11 were located in the centre of the field. The open field was positioned on a 700 mm-high table.

Each individual rat was placed into the centre of the apparatus and for 5 minutes, every 3 seconds the number of the square it was occupying and the type of behaviour it was

engaged in (walking, rearing, grooming, and immobility) were manually recorded. At the end of the trial, the number of faecal boluses left by the rat in the apparatus were also counted. High numbers of faecal boluses are often viewed as an indication of high anxiety (Archer, 1973). Time spent in the centre of the field and the outer segments as well as the number of transitions from one square to another were also recorded. Increased locomotor activity and occupation of the centre indicates lower anxiety, while less activity and a preference for the edges are indicators of higher anxiety levels.

2.5.2 Light/dark Box

The light/dark box has been widely used to measure anxiolytic effects of drugs and HP in particular (M. Bourin & Hascoet, 2003; Flausino et al., 2002; Hascoet & Bourin, 1998). It is useful for assessing rodents' emotional reactivity by exploiting their tendency to explore novel environments while avoiding illuminated areas (Hascoët, Bourin, & Dhonnchadha, 2001; Hughes, Desmond, & Fisher, 2004). Anxiety in this apparatus is measured by observing the number of entries into and occupation of a light versus dark chamber.

This apparatus consisted of a clear-varnished wooden box, 640 mm in length, 310 mm in width, and 230 mm in height. It comprised a dark compartment and an illuminated aversive compartment (both 300 mm long x 200 mm wide x 300 mm high) separated by a wall with an opening (100 mm x 100 mm) and a removable slide (120 mm wide x 150 mm high) that enabled access to both chambers. The dark compartment was covered by a hinged wooden lid, whereas the light side had a hinged clear Perspex lid which allowed light into it.

Each rat was placed in the dark compartment of the apparatus with the lid shut and the partition slide closed. When the trial started, the middle slide was lifted allowing the rat free access to both compartments. During a 5-minute trial, the latency of first emerging into the

light compartment, the number of entries into each compartment, and the occupation (number of 3-second observations) of the light compartment were recorded. The most reliable and useful response for assessing anxiolytic-like activity action is the time spent in the light compartment (M. Bourin, 2015) .

2.5.3 Y Maze

The Y maze test is able to measure both anxiety levels and short-term memory of rats (Aitchison & Hughes, 2006; Hughes, 2004). The apparatus comprised a wooden un-painted Y maze covered by a clear Perspex lid. It was 130 mm wide and 150mm wide, and consisted of a 300 mm-long stem and two arms with an angle of 120° between them. The arms were each 460 mm long. Each arm contained a removable black or white painted aluminium insert that is 400 mm long and 147 mm high. The stem was 160 mm long with a sliding partition at the proximal end for restricting the rat's access to the rest of the maze i.e., the start area.

Testing began with an acquisition trial during which each rat was placed in the Y maze for a 5-minute period of free exploration with one arm containing a black insert, and the other containing a white insert. After this, the rat was removed and kept in a holding cage for approximately 30 seconds while both inserts were replaced with cleaned and disinfected black inserts. Then the rat was placed in the start area of the stem for a retention trial. This involved a 3-minute period of free exploration following removal of the the sliding partition. Responses recorded were the first entry of an arm and the total entries into and the number of 3-second observations in each. These data enabled later calculation of the percent entries into and percent observations in the arm that had changed from white to black i.e., the novel arm.

2.5.4 Elevated Zero Maze

The elevated zero maze (EZM) is a test designed to measure anxiety by exploiting the natural tendencies for rats to explore a novel environment while also minimising time spent

in open or elevated areas. The apparatus is a reconstruction of the elevated plus maze, and is designed to eliminate vague interpretations of time spent in the central square of the plus maze (Shepherd, Grewal, Fletcher, Bill, & Dourish, 1994). This is achieved by a smoother transition from closed to open areas thereby allowing the rat to walk easily from one to the other. The EZM was 650 mm from the floor and was supported by four metal legs to provide stability. It consisted of a 410 mm diameter circular platform with a track width of 45 mm and with two open and two enclosed areas facing each other. The corridor length of the enclosed areas was 740 mm with 260 mm high walls. The open-area corridors were of the same dimensions but were only fenced with 10 mm high edges.

The test began with the rats being placed in an enclosed area. The latency of first emergence into an open area was recorded. Then for 5 minutes, the number of entries into each area was recorded along with the number of 3-second observations that the rat was seen in each.

2.6 Withdrawal Testing

Two separate open-field tests were performed at the 72 hours HP treatment free period and the two-week HP treatment free period respectively. The measures recorded and procedures followed the same process as described above for the open field test. The rats' exploratory behaviours including transitions, rearing, and the centre square occupancy were recorded as previous studies have used these measures to assess withdrawal effects of nicotine, amphetamine, and progesterone (Hitzemann et al., 1977; Irvine et al., 2001; Löfgren et al., 2006). These investigations found that the decreases in locomotor activity (transitions and rearing) was indicative of an anxiogenic effect showing increased anxiety on withdrawal from the substances studied.

Results

Separate 4 x 2 (dose x sex) Analyses of Variance (ANOVA) were performed on each response recorded in the four types of apparatus to evaluate the effects of chronic treatment with HP tincture, sex, and sex x treatment interactions. Additional ANOVAs were performed on two testing periods (HP treatment and withdrawal) for each type of apparatus. For post hoc comparisons, the Fisher's Least Significant Difference (LSD) test ($p<0.05$) was performed when appropriate. The HP treatment results are presented using tables and graphs with means and standard error of the means (SEM's) displayed.

3.1 Treatment Effects Data Analysis

Table 2 shows the means and SEM's as well as the ANOVA results for dose and sex effects of all measures of the open field, light dark box, y maze, and elevated zero maze.

3.1.1 Open Field Measures

3.1.1.1 Walking

The main effect of HP dose on walking was not significant ($F (3, 66) = 0.43$, $p = 0.73$). However, a main effect of sex differences for walking was reported ($F (1, 66) = 23.82$, $p < 0.001$). Results of female rats in all groups showed a significantly higher rate of walking than the male rats. There was no significant main interaction effect for dose and sex for walking.

3.1.1.2 Rearing

These was no significant main effect of HP dose on rearing ($F (3, 66) = 0.82$, $p = 0.49$). A main effect of sex differences was found to be significant ($F (1, 66) = 10.76$, $p < 0.001$). Female rats from all dose groups showed significantly higher rearing behaviour compared to the male rats. There was no significant interaction effect for dose and sex ($F (3, 66) = 0.98$, $p = 0.41$).

3.1.1.3 Grooming

For the grooming behaviour results, the main effect of HP dose was not found to be significant ($F(3, 66) = 0.52$, $p = 0.67$) nor was the main effect of sex differences ($F(1, 66) = 1.50$, $p = 0.22$). There was no main effect for dose and sex interaction ($F(3, 66) = 0.59$, $p = 0.63$).

3.1.1.4 Immobility

There was no significant main effect of HP dose on immobility ($F(3, 66) = 0.94$, $p = 0.43$). However a main significant effect of sex was found ($F(1, 66) = 25.96$, $p < 0.001$), indicating that male rats from all dose groups had higher immobility rate than the female rats. No significant interaction effect was found for dose and sex ($F(3, 66) = 0.94$, $p = 0.43$).

3.1.1.5 Faecal Boluses

The main effect of HP dose on faecal boluses was significant ($F(3, 66) = 4.55$, $p < 0.01$). The post hoc test revealed significantly higher amount of faecal blouses for the high dose group compared to the other dose groups, $p < 0.05$. In addition, a significant main effect of sex differences was reported ($F(1, 66) = 16.16$, $p < 0.001$) showing that male rats from all dose groups produced significantly higher number of faecal boluses. There was no significant main interaction effect for dose and sex ($F(3, 66) = 1.18$, $p = 0.33$).

3.1.1.6 Transitions

For transitions, results showed no significant main effect for HP dose ($F(3, 66) = 0.79$, $p = 0.51$). However a main effect in sex differences was found ($F(1, 66) = 35.12$, $p < 0.001$) indicating that the female rats from all groups had significantly more transitions compared to the male rats. No significant main interaction effect was found for dose and sex ($F(3, 66) = 2.64$, $p = 0.06$).

3.1.1.7 Centre Squares Occupancy

Results for the centre squares occupancy showed a significant main effect of HP dose (F (3, 66) = 3.94, p < 0. 01). As shown in Figure 1, the post hoc test revealed significantly higher centre squares occupancy rate for the low dose group compared to the control and the medium dose groups. However, there was no evidence for significant main effect of sex difference (F (1, 66) = 1.74, p = 0.19) or for the dose and sex interaction (F (3, 66) = 0.70, p = 0.57).

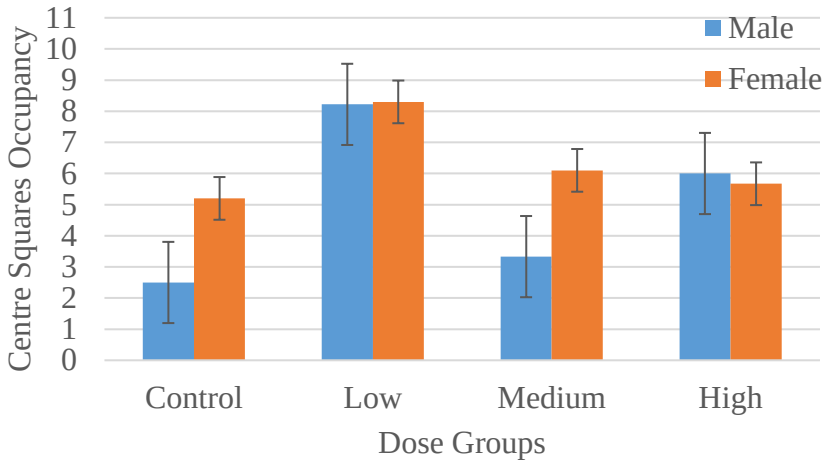

Figure1. Mean centre square occupancy for male and female rats in the open field

3.1.1.8 Outer Squares Occupancy

For the outer squares occupancy, there were no significant main effect of HP dose (F (3, 66) = 2.48, p = 0.07), sex difference (F (1, 66) =2.44, p =0.12), nor for the interaction between dose and sex (F (3, 66) = 1.27, p = 0.29).

3.1.2 Light/dark Box Measures

3.1.2.1 Emergence Latency

For emergence latency, there were no significant main effect of HP dose ($F(3, 66) = 1.63$, $p = 0.20$); sex difference ($F(1, 66) = 0.04$, $p = 0.84$); nor for the interaction between dose and sex ($F(3, 66) = 0.76$, $p = 0.52$).

3.1.2.2 Light Side Entries

The main effect of HP dose on light side entries was reported to be significant ($F(3, 66) = 3.31$, $p < 0.05$). Further data analysis revealed that the low dose group had significantly higher light side entries compared to the control and medium dose groups. In addition, the difference on the light side entries between the low dose group and high dose group is tending towards significance with $p = 0.08$. Figure 2 presents the comparison of the mean difference in entries among the four dose groups for both male and female rats. There were no main effects of sex difference ($F(1, 66) = 0.99$, $p = 0.32$) and dose x sex interaction ($F(3, 66) = 0.44$, $p = 0.73$).

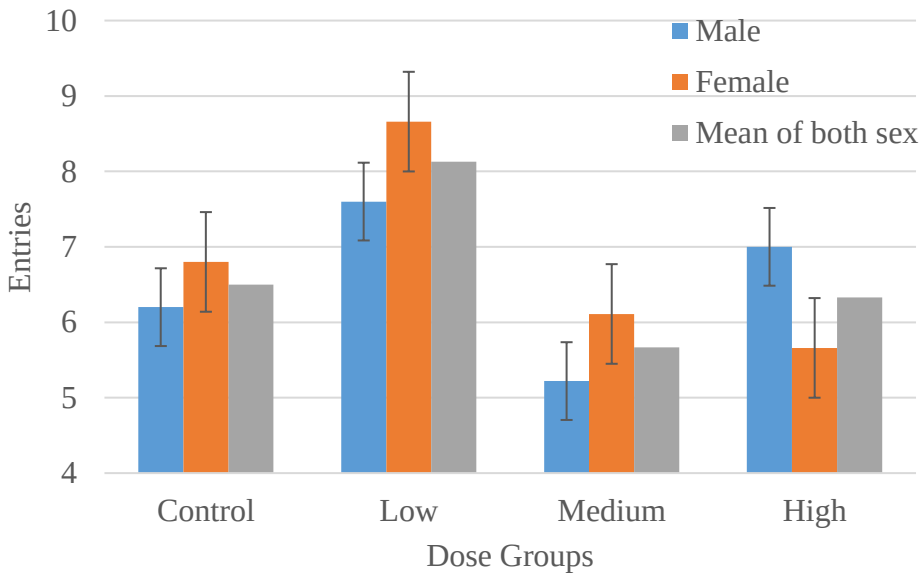

Figure 2. Mean entries of the light side for both male and female rats in the light dark box

3.1.2.3 Light Side Occupancy

For the light side occupancy, there was a significant main effect of HP dose ($F(3, 66) = 3.55$, $p < 0.05$). Rats from the low dose group had significantly higher light side occupancy rate than the control, medium, and high dose groups. There was no significant main effect of sex difference ($F(1, 66) = 1.86$, $p = 0.18$), and the interaction between dose and sex was found to be not significant ($F(3, 66) = 0.78$, $p = 0.51$).

3.1.2.4 Dark Side Entries

The main effect of HP dose on dark side entries was significant ($F(3, 66) = 3.27$, $p < 0.05$). Similar to the light side entries, results showed that the low dose group showed significantly higher entries rate compared to the control and medium dose groups, as which was trending towards significance with $p = 0.07$ when compared to the high dose group. No significant main effect was found for sex difference ($F(1, 66) = 1.53$, $p = 0.22$), nor the dose and sex interaction effect ($F(3, 66) = 0.32$, $p = 0.81$).

3.1.3 Y Maze Measures

Data for the three male rats (each from the control, low dose, and medium dose groups) were excluded from the analysis due to immobility from unknown causes. Overall data analysis showed no significant main effect for HP dose, sex, and dose x sex interaction for all of the Y maze measures including the percentage of novel arm entries and occupancy, and the entries into both arms.

3.1.3.1 % Novel Arm Entries

There was no main effect of HP dose ($F(3, 63) = 0.44$, $p = 0.73$), sex differences ($F(1, 63) = 1.41$, $p = 0.24$), nor the interaction between dose and sex ($F(3, 63) = 0.99$, $p = 0.40$) on the measure of the percentage of novel arm entries.

3.1.3.2 % Novel Arm Occupancy

Similar to novel arm entries, results of the percentage of novel arm occupancy did not indicate a significant main effect of HP dose ($F(3, 63) = 1.31$, $p = 0.28$), sex differences ($F(1, 63) = 2.37$, $p = 0.13$), nor the dose and sex interaction ($F(3, 63) = 0.21$, $p = 0.88$).

3.1.3.3 Entries into Both Arms

Results of both arms entries showed no significant main effect in HP dose ($F(3, 63) = 0.78$, $p = 0.51$), sex difference ($F(1, 63) = 0.02$, $p = 0.90$), nor the dose by sex interaction ($F(3, 63) = 0.88$, $p = 0.45$).

3.1.4 Elevated Zero Maze

For the Elevated Zero Maze, data of the four male rats (one from the control group, two from the low dose group, and one from the medium dose group) were excluded due to failure to emerge and failure to complete experimental procedures.

3.1.4.1 Emergence Latency

A significant main effect of the emergence latency of the HP dose was found ($F(3, 62) = 4.79$, $p < 0.01$) showing that the rats from the high dose group took significantly longer time entering to the open area. Result also showed a significant main effect in sex difference ($F(1, 62) = 14.72$, $p < 0.001$) as well as the dose and sex interaction ($F(1, 62) = 3.12$, $p < 0.05$). Further analysis revealed that only male rats from the high dose group took significantly longer time to emerge than the other dose groups ($F(3, 27) = 3.69$, $p < 0.05$). There was no significant differences among the dose groups for female rats ($F(3, 35) = 0.65$, $p = 0.59$).

3.1.4.2 % Open Area Entries

For the percentage of open arm entries, results showed no significant main effect of HP dose (F (3, 62) = 1.09, p = 0.36), sex difference (F (2, 62) = 0.33, p = 0.57), nor for the dose by sex interaction (F (3, 62) = 1.36, p = 0.27).

3.1.4.3 % Open Area Occupancy

There was no significant main effect of HP dose for the percentage of open area occupancy (F (3, 62) = 1.79, p = 0.16). However the main effect of sex was significant (F (1, 62) = 10.39, p < 0.01). Figure 3 shows that female rats occupied the open areas significantly longer than the male rats. No significant main effect of dose and sex interaction was found (F (3, 62) = 1.21, p = 0.31).

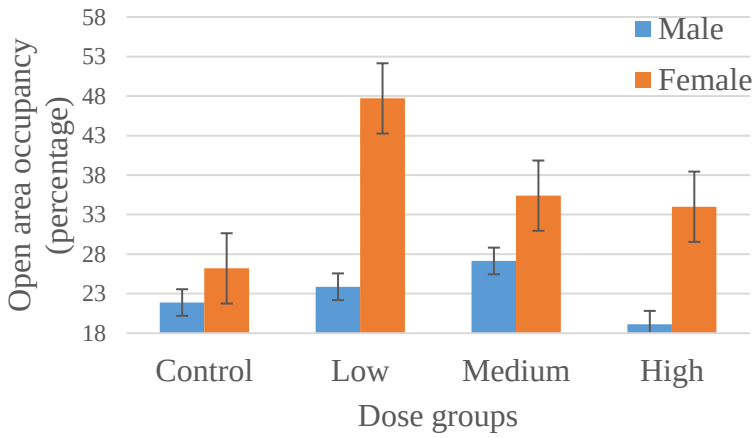

Figure 3. Open area occupancy for male and female rats in the elevated zero maze

3.1.4.4 Closed-area Entries

Result showed no significant main effect of HP dose on closed-area entries (F (3, 62) = 0.88, p = 0.46). A main effect of sex difference was reported (F (1, 62) = 16.25, p < 0.001) indicating that the female rats had a significantly higher entry rates to the closed-areas compared to the male rats. Results showed no significant main effect of the dose and sex interaction (F (3, 62) = 0.36, p = 0.78).

Table 2. *Mean (± S.E.M, in brackets) values of all measures of the open field, light dark box, y maze, elevated zero maze, and ANOVA results of dose and sex main effects*

Measure	Groups				F (3, 66)	Sex of the rats		F (1, 66)
	Control (0mg/kg) n = 20	Low (250mg/kg) n = 19	Medium (500mg/kg) n = 19	High (750mg/kg) n = 16		Males	Females	
Open field								
Walking	32.20 (1.10)	33.26 (0.87)	31.63 (1.10)	34.13 (0.74)	0.43	28.34 (0.89)	36.69 (0.79)	23.82***
Rearing	30.90 (1.31)	31.63 (1.12)	28.21 (1.37)	27.88 (0.61)	0.82	25.91(1.24)	33.18(0.92)	10.76***
Grooming	8.45 (0.82)	7.42 (0.79)	5.84 (0.62)	8.43 (0.10)	0.52	6.54 (0.71)	8.38 (0.87)	1. 50
Immobility	28.40 (2.05)	27.68 (1.86)	34.32 (2.38)	29.56 (1.33)	0.94	39.17 (2.02)	21.74 (1.29)	25.96***
Faecal boluses	0.80 (0.17)	0.89 (0.16)	1.21 (0.23)	2.50 (0.27)	4.55**	2.06 (0.24)	0.62 (0.16)	16.16***
Transitions	43.45 (1.64)	45.84 (1.40)	41.21 (1.91)	42.06 (1.32)	0.79	35.09 (1.28)	50.46 (1.33)	35.12***
Centre squares occupancy	3.85 (0.38)	8.26 (0.67)	4.79 (0.36)	5.81 (0.50)	3.94**	4.89 (0.61)	6.33 (0.42)	1.74
Outer squares occupancy	65.5 (1.36)	54.89 (1.52)	61.37 (1.53)	59.13 (1.46)	2.48	63.06 (1.64)	57.90 (1.35)	2.44
	n = 20	n = 19	n = 19	n = 16	F (3, 66)			F (1, 66)
Light dark box								
Emergence latency (s)	15.81 (1.42)	14.95 (1.41)	29.60 (4.72)	16.74 (2.28)	1.63	20.23 (3.08)	18.52 (2.65)	0.04
Light side entries	6.40 (0.19)	8.16 (0.21)	5.89 (0.33)	6.75 (0.31)	3.31*	6.49 (0.27)	7.08 (0.29)	0.99
Light side occupancy	31.70 (1.67)	42.05 (1.03)	30.05 (1.89)	29.75 (1.39)	3.55*	35.71 (1.72)	31.54 (1.51)	1.86
Dark side entries	6.10 (0.20)	7.79 (0.20)	5.63 (0.32)	6.38 (0.29)	3.27*	6.11 (0.26)	6.79 (0.28)	1.53

Main significant effect * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 2 continued

Measure	Groups				F (3, 63)	Sex of the rats		F (1, 63)
	Control (0mg/kg) n = 19	Low (250mg/kg) n = 18	Medium (500mg/kg) n = 18	High (750mg/kg) n = 16		Males	Females	
Y maze								
% Novel arm entries	61.38 (2.27)	59.33 (1.94)	57.37 (3.23)	66.47 (2.24)	0.44	58.01 (2.35)	63.44 (2.52)	1.41
% Novel arm occupancy	10.18 (0.72)	15.93 (0.99)	15.37 (1.69)	13.02 (0.95)	1.31	11.51 (0.82)	15.29 (1.35)	2.38
Entries into both arms	4.47 (0.30)	5.06 (0.23)	4.00 (0.31)	4.88 (0.27)	0.78	4.59 (0.23)	4.59 (0.31)	0.02
	n = 19	n = 18	n = 17	n = 16	F(3, 62)			F (1, 62)
Elevated zero maze								
Emergence latency (s)	38.73 (3.84)	22.81 (2.00)	35.40 (2.84)	64.27 (8.53)	4.79**	57.46 (6.61)	25.46 (2.34)	14.72***
% Open area entries	50.47 (0.14)	51.79 (0.47)	51.09 (0.17)	51.34 (0.28)	1.09	51.31 (0.41)	51.04 (0.16)	0.33
% Open area occupancy	24.16 (1.76)	37.11 (2.36)	32.00 (2.50)	27.50 (1.69)	1.79	22.97 (2.20)	35.87 (1.86)	10.39**
Closed-area entries	6.79 (0.44)	8.22 (0.40)	7.24 (0.44)	6.69 (0.42)	0.88	5.48 (0.36)	8.64 (0.40)	16.25***

Main significant effect * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

3.2 Withdrawal Effects Data Analysis

First of all, a one-way between subjects ANOVA was conducted to compare the effect of HP treatment time and treatment withdrawal conditions. The time period was divided into during treatment, treatment acute withdrawal (72 hours), and treatment chronic withdrawal (14 days). Measures for exploratory behaviours including transitions, rearing, and centre squares occupancy were analysed. Results showed a significant main effect among the three time periods for all of these measures: transitions ($F(2, 210) = 16.83$, $p < 0.001$), rearing ($F(2, 210) = 14.63$, $p < 0.001$), and centre squares occupancy ($F(2, 210) = 9.02$, $p < 0.001$). Table 3 shows the means and SEM's as well as the ANOVA results for dose and sex effects of all measures of the open field during acute and chronic withdrawal periods. As shown by Figure 4, the rats' transition rate was significantly higher during the HP treatment time period compared to the HP treatment free periods ($p < 0.01$). The transition rate was significantly higher for the rats tested during the chronic HP treatment withdraw period compared to those during the acute treatment withdrawal period ($p < 0.01$). Furthermore, Figure 5 shows that the highest rearing behaviour occurred during the HP treatment period and it was significantly higher than the acute HP treatment free period ($p < 0.01$). However it was not significantly higher than the chronic HP treatment free period. Figure 6 presents that rats under HP tincture treatment had significantly higher centre squares occupancy compared to the HP treatment free periods ($p < 0.01$). Consistent to the results of transitions, the rats' centre squares occupancy rate was significantly higher during the chronic withdrawal period compared to the acute withdrawal time period ($p < 0.01$).

Table 3.

Mean (± S.E.M, in brackets) values of all measures of the open field during acute and chronic withdrawal periods

Open field	Groups				F (3, 66)	Sex of the rats		F (1, 66)
	Control (0mg/kg) n = 20	Low (250mg/kg) n = 19	Medium (500mg/kg) n = 19	High (750mg/kg) n = 16		Males	Females	
Acute withdrawal								
Transitions	27.15 (1.62)	35.32 (1.50)	28.16 (1.41)	29.88 (1.28)	2.25	22.43 (1.05)	36.97 (1.36)	35.16 ***
Rearing	18.15 (1.28)	23.68 (1.20)	21.42 (1.19)	19.13 (0.92)	1.20	16.63 (0.98)	24.21 (1.18)	11.08 ***
Centre squares occupancy	2.80 (0.28)	4.79 (0.42)	2.74 (0.25)	3.44 (0.37)	2.06	2.57 (0.28)	4.21 (0.37)	6.09 *
Chronic withdrawal								
Transitions	34.90 (1.65)	38.58 (1.98)	37.00 (1.55)	35.38 (1.43)	0.34	27.20 (1.19)	44.82 (1.29)	44.00 ***
Rearing	27.75 (1.42)	32.26 (2.09)	26.21 (1.26)	27.44 (0.73)	0.83	23.86 (1.43)	32.56 (1.36)	10.40 **
Centre squares occupancy	3.05 (0.38)	4.79 (0.49)	3.68 (0.49)	2.56 (0.27)	1.43	2.94 (0.47)	4.10 (0.37)	2.49

Main significant effect * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Further investigation showed that the main effect of HP dose was not significant for transitions ($F(3, 210) = 1.54$, $p = 0.20$) and rearing ($F(3, 210) = 1.85$, $p = 0.14$). However a significant main effect of HP dose was reported for centre squares occupancy ($F(3, 210) = 6.23$, $p < 0.001$). Results showed that low dose group had significantly higher rate of centre squares occupancy than the other dose groups throughout the three testing periods. In terms of the time x dose interaction effect, results yielded no significance for these measures: transitions ($F(6, 210) = 0.27$, $p = 0.95$), rearing ($F(6, 210) = 0.37$, $p = 0.90$), and centre squares occupancy ($F(6, 210) = 0.83$, $p = 0.54$). However patterns of the group observations illustrate difference among dose groups across different time periods. Figure 4, Figure 5, and Figure 6 show the means of transitions, rearing, and centre square occupancy for the control and dose groups across treatment, acute withdrawal, and chronic withdrawal time periods. Rats from the low dose group have the highest transition rate and centre occupancy compared to the other groups regardless of different testing time periods.

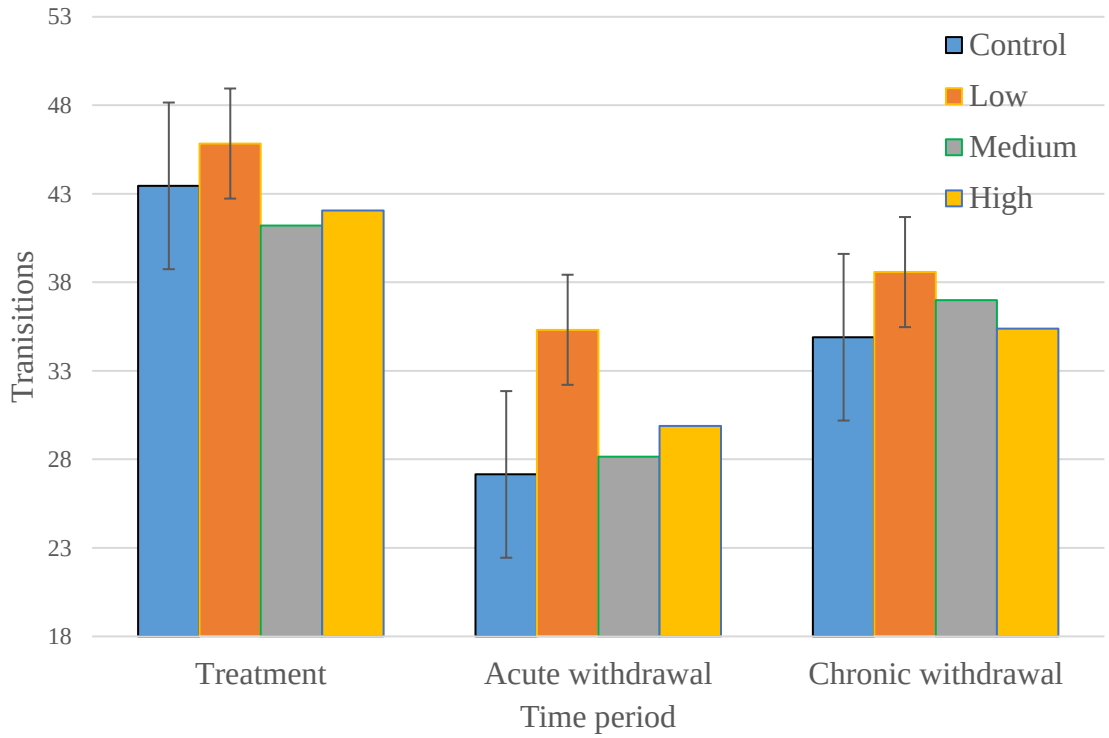

Figure 4. Means of the transitions of each dose group across three time periods

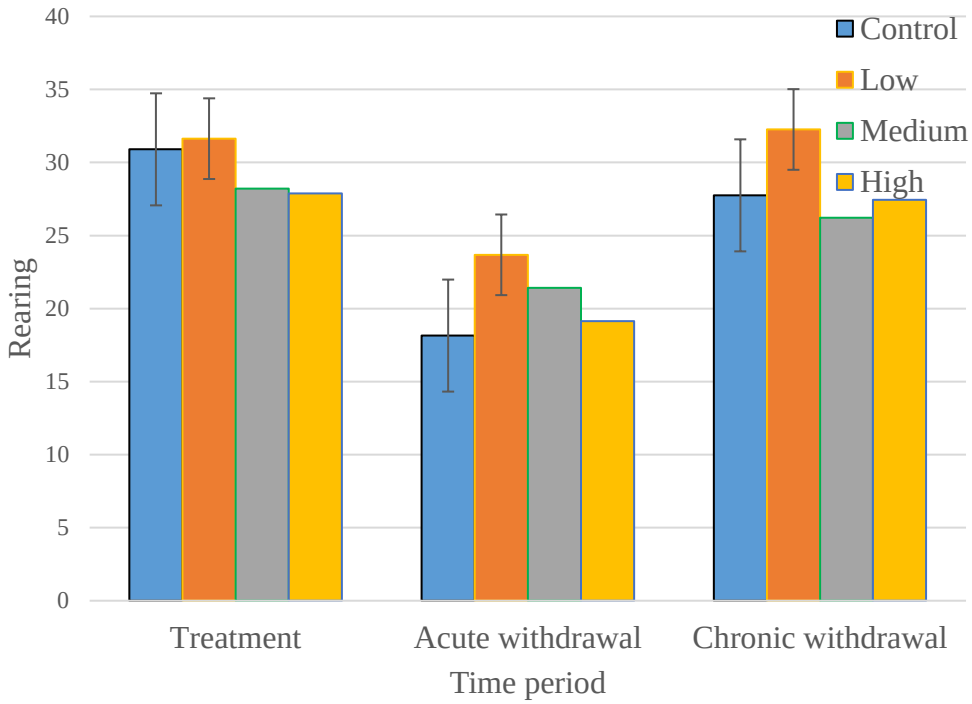

Figure 5. Means of the rearing behaviour of each dose group across three time periods

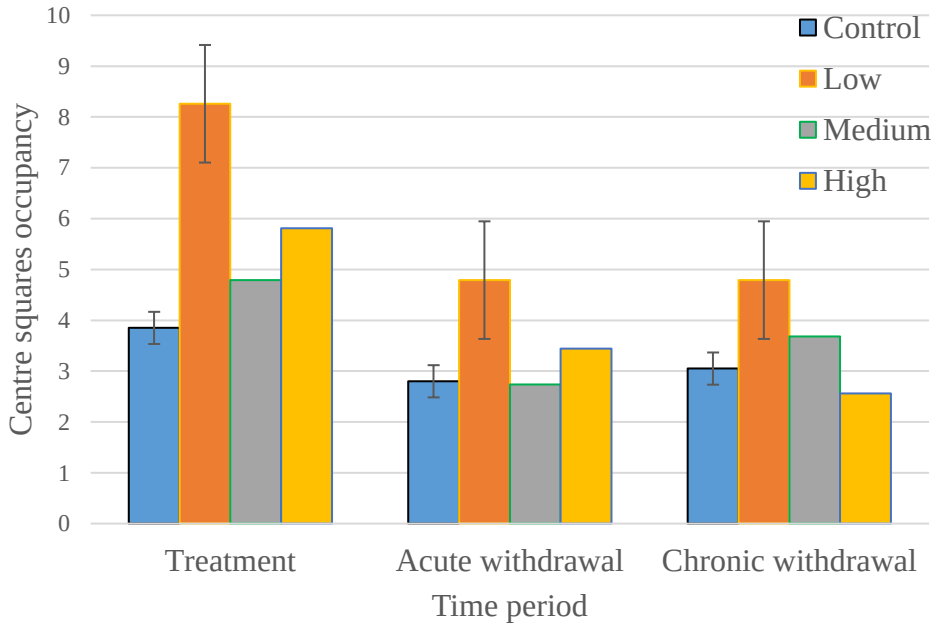

Figure 6. Means of the centre squares occupancy for each dose group across three time periods

Furthermore, separate one-way between subjects ANOVA was performed to investigate possible withdrawal effects during a 72-hour and 14-day HP treatment free period in an open field maze respectively. Table 3 displays the means, SEM's, and the ANOVA results for dose and sex effects of the measures of the open field tests during acute and chronic withdrawal periods.

3.2.1 Acute Withdraw (72 hours)

3.2.1.1Transitions

During the acute HP treatment free period, no significant main effect was found of HP dose on transitions ($F (3, 66) = 2.25$, $p = 0.09$). There was a main effect of sex differences ($F (1, 66) = 35.16$, $p < 0.001$) indicating that the female rats crossed the squares a lot more than the male rats. However the main interaction effect between the dose and sex was not found ($F (3, 66) = 1.60$, $p = 0.20$).

3.2.2.2 Rearing

There was no significant main effect of the HP dose groups of the amount of rearing ($F (3, 66) = 1.20$, $p = 0.32$). Results showed a significant main effect in sex difference ($F (1, 66) = 11.08$, $p < 0.001$) with significantly higher rate of the rearing behaviour in female rats. There was no significant main effect in dose and sex interactions ($F (3, 66) = 0.83$, $p = 0.48$).

3.2.2.3 Centre Squares Occupancy

Results for the centre squares occupancy indicated no significant main effect of HP dose ($F (3, 66) = 2.06$, $p = 0.11$). However post hoc test revealed that rats from the low dose groups had significantly higher centre square occupancy than the control and medium dose groups ($p < 0.05$). In addition, a main effect of the sex difference was found to be significant ($F (1, 66) = 6.08$, $p < 0.05$) showing that female rats had significantly higher rate of centre

area occupancy compared to the male rats. No significant main effect of dose and sex interaction was found (F (3, 66) = 0.40, p = 0.75).

3.2.2 Chronic withdraw (14 days)

3.2.2.1 Transitions

For the 14-day chronic HP treatment free period, there was no significant main effect of HP dose for transitions (F (3, 66) = 0.34, p = 0.80). A significant main effect in sex difference was found (F (1, 66) = 44.00, p < 0.001) showing that female rats had significantly higher rate of transitions than male rats. There was no significant main effect in the interaction between sex and dose groups (F (3, 66) = 1.69, p = 0.18).

3.2.2.2 Rearing

No significant main effect was found for the HP dose on rearing (F (3, 66) = 0.83, p = 0.48). However results showed that female rats had a significantly higher rate of rearing compared to the male rats (F (1, 66) = 10.40, p < 0.01) as shown in Figure 7. Although the pattern fluctuates among the dose groups for male and female rats, there was no significant main effect in dose and sex interaction.

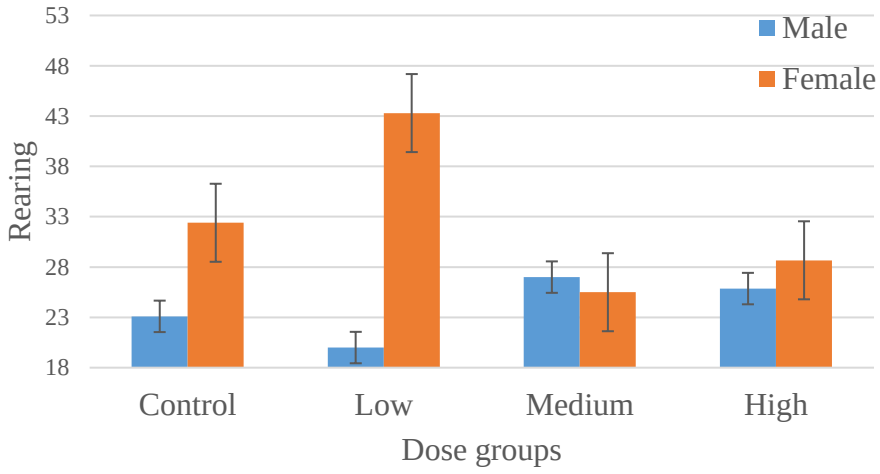

Figure 7. Means of rearing in the open field test during chronic withdraw for both male and female rats

3.2.2.3 Centre Squares Occupancy

For centre squares occupancy during the chronic HP treatment free period, results showed no significant main effect of HP dose ($F(3, 66) = 1.43$, $p = 0.24$) and sex difference ($F(1, 66) = 2.49$, $p = 0.12$). However a significant interaction between dose and sex was found ($F(3, 66) = 3.96$, $p < 0.01$). Figure 8 shows that male and female rats from the low dose and high dose groups had the opposite occupancy compared to these from the medium dose groups.

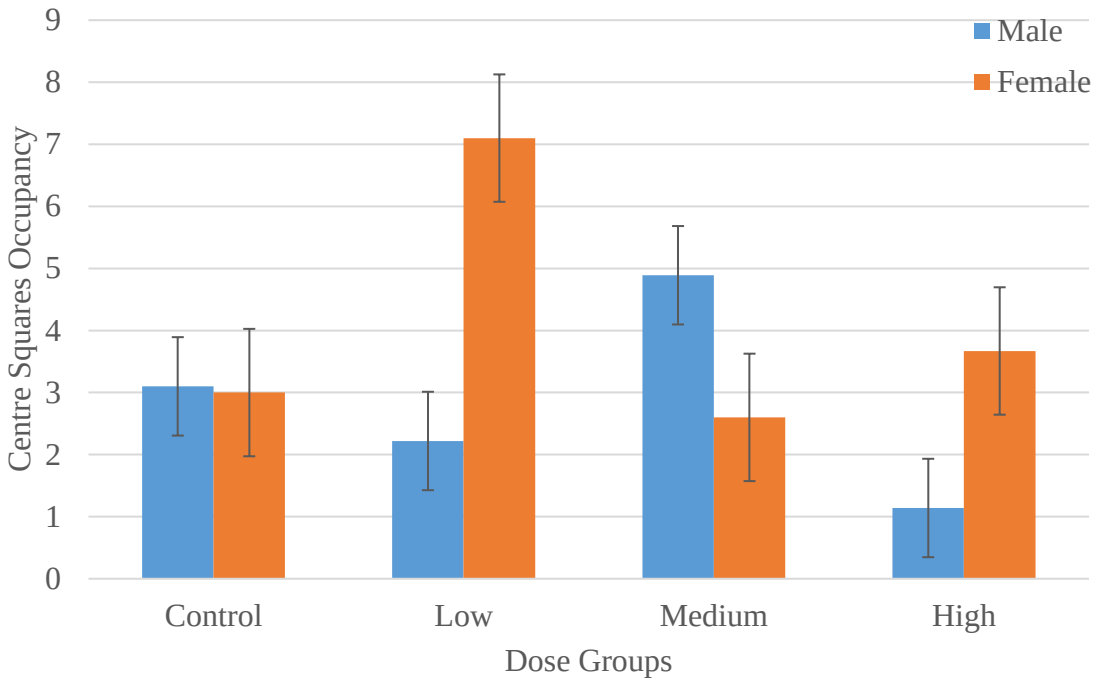

Figure 8. Means of centre square occupancy in the open field test during chronic withdraw for both male and female rats

Discussion

4.1. Summary

The goals of this research were to (1) investigate if there is evidence to support HP's anxiolytic effect by comparing rats' anxiety responses from a control group and three dose groups; (2) assess if there are differences in the behavioural responses among the dose groups, and to detect which dose(s) produce the greatest reduction of anxiety; (3) assess sex differences in how each dose affect anxiety; (4) detect if there are withdrawal effects upon the termination of HP treatment during two time periods (acute and chronic withdrawal). To achieve these aims study, observations of rats responses in four behavioural tests were analysed using 4 x 2 (dose x sex) factorial ANOVA as well as Fisher's LSD post hoc tests.

Overall, the present study shows some support for the prediction that HP tincture has an anxiolytic effect by reducing rats' anxiety responses in measures of centre squares occupancy and faecal boluses from the open field; light side entries/occupancy, and light side entries from the light/dark box. Apart from no observable effect in the Y maze test; the data analyses showed variation in the rats' responses among the treatment and control groups indicating anxiolytic effects of the HP tincture treatment. Overall the results suggested that the low dose (250mg/kg) group displayed lower anxiety responses compared to other dose groups and the control group. Furthermore, there was evidence that male and females responded differently across measures of the open field and elevated zero maze. Evidence for dose and sex interaction effect was obtained on emergence latency of the elevated zero maze. The emergence latency was used as a measure for examining increased anxiety responses (Cook, Crounse, & Flaherty, 2002). This finding indicated a higher level of anxiety for the male rats from the high dose (750mg/kg) group as they took significantly longer time to emerge into the open areas. Withdrawal tests showed a higher anxiety reduction rate during

the treatment period compared to the treatment free periods. In addition, the low dose (250mg/kg) for both male and female rats appeared to produce lower anxiety than other doses as presented by increase locomotor activates and explorative behaviour such as number of transitions and percentage of time spent occupying the centre squares. These findings support the HP tincture's anxiolytic effect, and the low dose (250mg/kg) as a potential optimal dose for both male and female rats.

4.2 Anxiolytic and Dose Effects

Different from Vandenbogaerde et al. (2000)'s HP extract study of reporting the anxiolytic effects (presented by increase number of transitions, rearing, and total pathway length) on the open field, this study found a higher centre squares occupancy with a specific dose. This evidence from the open field test showed response variation among the dose and control groups. A body of literature stated that rats are social animals and prefer to occupy small spaces rather than large open areas. The open field test is a standard neophobic anxiety measure in rodents. According to rats' natural tendency to occupy the periphery of an open area, the open field test indicates reduced anxiety by making more entries into the central area and more exploratory behaviour in the apparatus (Hiroi & Neumaier, 2006; Prut & Belzung, 2003). This study reported the anxiolytic effects and a potentially efficient dose level of the HP tincture treatment with supportive evidence of having the highest centre occupancy rate for the rats from the low dose (250mg/kg) group compared to the control and medium dose (500mg/kg) groups. In addition, a large number of faecal boluses is often viewed as an indication of higher level of anxiety (Archer, 1973). Results indicated that the HP high dose (750mg/kg) group produced significantly more faecal boluses representing an increased anxiety response. In summary, the open field test results showed highest anxiolytic effects for the low dose group, with possible increased adverse effects for the rats from the high dose group.

The light/dark box has been reported as a reliable measure to detect anxiolytic treatment effects in rodents (Crawley & Goodwin, 1980). Due to rats' natural aversions to a bright light and the conflict with their tendency to explore a new area, the measure of locomotor activity and time spent in the light compartment indicates a lower level of anxiety (Belzung, Misslin, Vogel, Dodd, & Chapouthier, 1987; Hascoët et al., 2001). The results from this study showed a significantly higher transition as well as the light side occupancy rate for the low dose group. This pattern of behaviour illustrates an anxiolytic effect of the low dose (250 mg/kg) of the HP tincture. This finding supports the previous research in finding the most efficient dose for treating anxiety by showing the HP treatment at a dose level of 250mg/kg enhanced number of transitions between the light and dark compartments (Flausino et al., 2002).

In terms of the y maze and elevated zero maze tests, there were no significant anxiolytic effects detected for different dose groups and the control group. However results of the elevated zero maze reported that the high dose group male rats took a longer period of time before emergence to the open area. Although this may be an indication of a higher anxiogenic state, however the emergence latency may not be an adequate measure for testing anxiogenic or anxiolytic drug effects (Matto, Harro, & Allikmets, 1997). Overall, the analysis revealed that the low dose (250mg/kg) had the highest anxiety reduction rate suggesting that this is the optimal dose for an anxiolytic effect.

4.3 Sex Differences

Significant sex differences were apparent in this study. Aligned with a previous mice study from Tucker, Fu, and McCabe (2016), findings of the present study also reported that female rats had increased activities levels compared to the male rats in the open field and zero

maze tests. However there was no evidence obtained for sex differences from the measures of the light/dark box, and the y maze.

In the open field test, sex differences were evident in a number of recorded measures including: the amount of transitions, walking, rearing, immobility, and faecal boluses. Female rats appeared to be more active as presented by higher counts for transitions, walking, and rearing. This finding is indicative of anxiolytic effects showing higher locomotor activities and more explorative behaviour (Hiroi & Neumaier, 2006; Prut & Belzung, 2003; Vandenbogaerde et al., 2000). In contrast, male rats displayed higher frequencies of immobility and the number of faecal boluses indicating higher levels of anxiety (Archer, 1973). The highest centre occupancy rate for the low dose group (250mg/kg) was apparent for both male and female rats in the open field test. Although there was one measure (transitions) suggesting a dose and sex interaction effect, this result did not reach statistical significance.

In the light/dark box, sex differences were not reported to be statistically significant. However, the response pattern revealed that the male rats from the high dose (750mg/kg) group had a higher entrance rate into the light compartment than the female rats from the high dose group, compared to a higher entry rate for females from all the other dose groups. This result might be indicative that each sex has a different dosage response level.

According to the elevated zero maze data, findings of sex differences were reported on open area occupancy and closed area entries which is similar to the previous experiment conducted by Tucker et al. (2016). This study also reported that female rats from the low dose group not only had significantly higher occupancy compared the females from other dose groups, but also than the male rats from the low dose group. This finding may suggest that the low dose level is the most effective dose for excreting anxiolytic effects for female rats.

As discussed previously, a dose and sex interaction was also shown on the measure of emergence latency for the male rats from the high dose group showing a possible higher anxiogenic state. Regardless of insufficient evidence obtained from the present study claiming an interaction between dose and sex, current results further support that female rats responded to the HP treatment differently from the male rats at a different dose level (Gray & Hughes, 2015).

4.4 Withdrawal

There is limited literature describing HP treatment withdrawal effects, and certainly not for HP tinctures. Behavioural measures analysed were decided according to the existing research on other substance studies using the open field test (Hitzemann et al., 1977; Löfgren et al., 2006). First of all, consistent with the first prediction of this study, anxiolytic effects of the HP tincture treatment was also supported by the open field withdrawal data analysis. For transitions, rearing, and centre area occupancy, there were significant differences among the HP treatment period, acute withdrawal, and the chronical withdrawal period. The results suggest significant anxiety reduction responses for the rats that were tested during the treatment time compared to the treatment free periods. Furthermore, data from the chronic withdrawal period showed a significantly reduced anxiety response compared to the acute withdrawal period which is supported by the significant increase in theses locomotor activities. Compared to the HP treatment and chronic withdrawal period, a withdrawal response was evident from the rats' anxiety-related reduced locomotor activities during the acute withdrawal period. This finding is consistent with the previous study from Beckman et al. (2000) that claimed the production of withdrawal effect from HP treatment that was similar to other antidepressants after one week of treatment discontinuation.

The results showed no statistical significance when the main effects of dose and time were further investigated. A reduction of exploration was suggested thereby indicating reduced anxiolysis during the treatment free periods. However a consistent pattern from Figure 4, Figure 5, and Figure 6 shows that rats' behaviours from the low dose (250mg/kg) group presents the highest anxiolytic effect across all of the three time frames. The findings further supported that the low dose level of 250mg/kg may have a superior anxiolytic effect compared to other doses (0, 500, 750 mg/kg).

Further data analysis for the acute and chronic withdraw time periods showed significant sex differences respectively that is consistent with the treatment period results. Nonetheless, neither the acute nor the chronic withdrawal period analyses indicated a dose or dose x sex interaction effect. The response pattern showed an adjustment period during the acute treatment withdrawal phase as presented by lower locomotor activities. Overall evidence did not show any significant adverse effects after the HP tincture treatment withdrawal.

4.5 Methodological Limitations

This study had some limitations. Firstly, the particular HP tincture used in this study is not the pure form of HP extract, such as the HP LI160 used in previous clinical trials (Flausino et al., 2002). While the aim of this study was to investigate the possible anxiolytic effect of the HP tincture on the current market, it should be noted that the treatment solution contained HP tincture (60% ethanol 1:2 tincture), plain tap water, and sucrose (to increase palatability and thus encourage drinking). Therefore, the combination of ethanol and 1% of sucrose might also influence the rats' behaviour. Ethanol withdrawal responses in rodents have been reported including hyperexcitability, stereotypic behaviour such as sniffing and grooming, as well as increased startle reflex and seizures (Adinoff, Majchrowicz, Martin, &

Linnoila, 1986; Little, Dolin, & Halsey, 1986). However, there was no evidence of such responses occurring in the present study. In addition, to control for any potential effect of sucrose, the control group had the same proportion of the sugar added to the drinking water. Even though there was no evidence of ethanol effects being important, it would be advisable for this to be controlled for in future research with HP tincture.

Another potential limitation of this study involved the choice of the different dose levels which were selected on the basis of previous research (Beijamini & Andreatini, 2003; Flausino et al., 2002; Grundmann et al., 2010; Kumar et al., 2000). Ideally, a form of the HP extract would have been used. However, this was not possible due to time constraints on the study and the inability to obtain such an extract from the only listed supplier (in Germany), in spite of numerous attempts. It is possible that the HP extracts used in clinical trials (such as LI160) is commercially available outside of Germany which could cause difficulties for research into HP extracts.

According to Bilia, Gallori, et al. (2002), two different alcoholic tinctures (40% and 60% v/v) of HP contained different levels of flavonol and hypericin. In a review, Linde, Berner, and Kriston (2008b) considered HP doses of between 240 and 1800 mg and concluded that the composition of the products can be very different depending on the plant selection, extract preparation process, and the use of solvents. These findings suggest that the level of effectiveness of the HP tinctures can be based on the production methods and processes. This may pose another limitation, as this study was conducted with only one type of HP tincture meaning that the results may not apply to all preparations. This issue is also apparent in the previous studies that formulated own HP treatment by using a dry form of HP extract under different trademarks (A. Kumar, R. Garg, & A. K. Prakash, 2010; Vandenbogaerde et al., 2000).

4.6 Implications and Future Research

This is the first study to investigate a HP tincture that is available on the commercial market. There has been considerable previous research on the antidepressant effects of HP extracts. However its anxiolytic potential has not been extensively studied, and sex difference in its effects have not been considered. In addition, there is a lack of effective dose information to assist in the use of HP products. This current study attempted to address some of these gaps.

HP products are a highly popular alternative herbal medicine that does not require a doctor's prescription and can be readily purchased by consumers. A recent review by Forsdike and Pirotta (2019) investigated the perception and use of HP extracts among general medical practitioners (GPs). The authors showed that GPs outside of Germany rarely recommended or prescribed HP products with poor knowledge of the herb and its effectiveness. The authors also expressed their concerns about HP self-medication in the absence of adequate clinical evidence with respect to HP extract preparations and doses. Therefore, the present study is a pioneer in this field aiming to provide useful information for health professionals and consumers.

In addition, there are possible herb-drug interactions of HP extracts when consumed with other medication. Cautions have arose when co-consumption occurs for the HP products and other medications. Finding have reported that the HP content, with > 1 mg hyperforin having a potential to cause serious drug interactions (Chrubasik‐Hausmann, Vlachojannis, & McLachlan, 2019). Furthermore, HP extract is known to interact with mainstream medication for managing anxiety and depression such as Alprazolam and Amitriptyline, as well as other prescription drugs including Warfarin, Cyclosporine, Digoxin, HIV protease inhibitors, anticonvulsants, triptans, and oral contraceptives (Mullaicharam & Halligudi,

2019). It is necessary for future research to further investigate different dose levels with different forms of the HP products, and their interactions with other medication.

In conclusion, the present study has provided some evidence supporting the anxiolytic effect of an HP tincture. This study has also found that the dose of 250mg/kg produced the greatest anxiolytic effect and the fewest withdrawal responses. Possible withdrawal responses were also evident during the acute HP tincture treatment withdrawal period thus suggesting an anxiogenic state. Overall, this is a significant first step in initiating research into popular herbal products such as HP extracts. Sex difference showing different levels of anxiety responses between male and female rats are also evident from the present study. Despite insufficient evidence to support an interaction effect between dose and sex, a pattern of different behavioural responses from male and female rats regarding to certain HP dose levels has been presented. It is important to recognise any effectiveness of the HP products, and to communicate and share the research findings with the health and mental health professionals. It is also recommended that consumers should seek specialists' advice when considering taking HP products with other medication especially in view of the lack of regulation and the lack of interest in and knowledge about HP products among GPs. More research into specific HP products and their anxiolytic effects in relation to various dose levels should be conducted with both male and female subjects.

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